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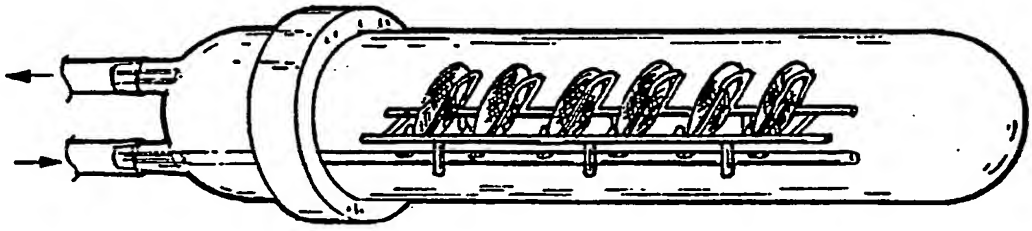

(57) Abstract

A process for producing secondary metabolites of viable plant cells, including the steps of: (a) preparing a support comprising a substantially uniform and porous matrix of inorganic material having a tensile strength of at least 500 MPa; (b) introducing a culture of viable plant cells into the pores of said matrix; (c) entrapping the plant cells by coating the matrix with a sol or colloidal suspension not interfering with the cell viability; (d) immobilizing the entrapped cells within the matrix with a reactive gas including a carrier gas saturated with volatile SiO_2 or organic modified SiO_2 precursors. The matrix may be a SiO_2 or inorganic oxide matrices, in which the weight ratio between cell load and inorganic material ranges between $1x10^{-4}$ and $1x10^{-2}$. The immobilized cells are not released in solution over a period of 6 months and maintain their viability while producing secondary metabolites.

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PROCESS AND ARTICLES FOR PRODUCING SECONDARY METABOLITES OF VIABLE PLANT CELLS IMMOBILIZED IN A POROUS MATRIX

The present invention relates to a process for continuously or discontinuously producing secondary metabolites of viable plant cells in porous and inorganic matrices.

The invention further relates to an article suitable for entrapping and immobilizing the plant cells in such a condition to maintain their viability for production of secondary metabolites.

The production of secondary metabolites on an industrial scale constitutes the main but not the only field of application of the present invention, since the process according to the invention and the immobilization obtained, as described in detail hereafter, can be advantageously used in any other equivalent field in which immobilized plant cells are used.

The most commonly used immobilization matrix is algal polysaccharide alginate, cross-linked with calcium ions as described in FEBS Lett. 103: 93-97 (1979) and 122: 312-316 (1980), Plant Cell Rep. 5: 302- 305 (1986), and Appl. Microbiol. Biotechnol. 30: 475-481 (1989). Other methods include entrapment with polyurethane foam, described in Biotechnol. Bioeng. 33: 293-299 (1989), 35: 660-667 (1990), Appl. Microbiol. Biotechnol. 33: 36-42 (1990), 37: 397-403 (1991) and 35: 382-392 (1991).

The use of porous and amorphous sol-gel derived silica for entrapment of viable cells has been described in cases of non-plant cells, reported in J. Biotechnol. 30: 197 (1993), J. Ceram. Soc. Jpn. 100: 426 (1992), Biochim.

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Biophys. Acta 276: 323 (1972), Chemistry of Materials 6: 1605-1613 (1994) and Angew, Chem, Int. Engl. 34: 301-303 (1995). These latter cannot be considered as methods actually suitable for applications to higher plant cells, which are severely poisoned under the experimental conditions reported for immobilization of bacteria and yeast cells. As for processes applied to, and claimed for, plant cell immobilization, they entail some problems: first of all, processes based on simple adhesion to the surface cannot properly be considered as immobilization, since cell reproduction and the increase in biological mass unavoidably cause release of cells in solution.

Polyurethane foaming matrices may cause severe transport limitations to and from immobilized cells. One important drawback of these host matrices is their poor mechanical stiffness, so that prolonged use for industrial production in practice does not appear feasible.

Immobilization in alginate beads allows direct contact of cells with the gel matrix, so that the cells are inevitably subjected to a high concentration of a variety of ions and organic compounds, causing negative physiological effects.

A main object of the present invention is therefore to overcome the above described disadvantages by means of a process which allows plant cells to be immobilized while maintaining their viability and avoiding the release of cells from the matrices, with free transport between the immobilized phase and the culture medium.

A further object of the present invention is to provide a process which may be repeated in standard

conditions with constant results.

Another object of the invention is to provide a host matrix with stiffness suitable to tolerate stress and share strain during production.

Still another object is to provide immobilization articles or matrices which, allowing free exchange of organic species and nutrients through the open pores, ensure increased intercellular contacts and consequently the potential for biochemical communications.

A further object of the invention is to provide a process which may be performed with industrial-scale devices and relevant production equipment.

These objects are achieved by a process comprising the steps of:

(a) providing a support comprising a substantially uniform and porous matrix of inorganic material having a tensile strength of at least 500 MPa;

(b) introducing a culture of viable plant cells into the pores of said matrix;

(c) entrapping the plant cells by coating the matrix with a sol or colloidal suspension not interfering with the cell viability;

(d) immobilizing the entrapped cells within the matrix with a reactive gas including a carrier gas saturated with volatile SiO_2 or organic modified SiO_2 precursors.

The employment of culture systems in which plant cells are immobilized on an inert support is now recognized as a means by which the environment of the cells can be manipulated simply and the yields of specific secondary metabolites increased over those of liquid-suspended cells,

thus allowing continuous production thereof.

The immobilization of plant cells obtained according to the invention covers a vast range of applications for the production of secondary metabolites, since the process is not limited to a single plant species and the coupling between the mechanical stiffness of the matrices with the porosity of deposited precursor-derived silica allows the application of this immobilized biosystem to heterogeneous phase production for large-scale industrial bioreactors.

The applicant has now surprisingly found - and this is one main aspect of the present invention - that it is possible to obtain immobilization of plant cells with maintenance of their viability.

The matrix may be a glass fiber fabric, a porous glass, ceramic, clay or similar inorganic material.

More preferably, the matrix may be a fabric or an agglomerate of inorganic fibres, which is impregnated with a gelling solution of SiO_2 precursors or similar materials to increase the stiffness thereof. For example, an ordinary glass fabric may be used having a fiber density between 100 and 700 mg/cm^2, which fabric is dipped in a gelling solution of $Si(OEt)_4$ and $CH_3SiH(OEt)_2$. The wet material is set aside for 15 days, developing a surface deposit of amorphous silica. The stiff fabric thus obtained may be cut into pieces of variable geometry.

In general, the glass fabric is preferably composed of fibers between 30 and 10 μm in diameter, with variable composition of the glass phase. The texture is compatible with the introduction of plant cells according to step (b). The concentration of $Si(OEt)_4$ and $CH_3SiH(OEt)_2$ used to

increase the stiffness and mechanical stress of the fibre matrix ranges between 10 and 100 g/dm^3 of nominal SiO_2. The solvent is preferably chosen among one or more of the following: ethanol, methanol, butanol, acetone, tetrahydrofuran, dimethylformamide. The solution contains a H_2O concentration ensuring hydrolysis of Si-OR groups, and is acidified with a nominal H^+ concentration ranging between $1x10^{-1}$ and $1x10^{-5}$ M. The viscosity suitable for step (a) preferably ranges between 0.2 and 100 Pas. The operation conducted with gelling solutions of species $Si(OR)_4$, $SiH_X(OR)_{4-X}$ and $SiX_X(OR)_{4-X}$, where x=1,2; R=alkyl or aryl, X=halide or alkyl, leads to the same results. Prepolymerized silicon derivatives of these species produce identical results.

Pieces of glass fabric with identical geometries may be assembled in a pile to become self-carrying after step (a).

The introduction of cells in accordance with step (b) is performed by simply shaking glass fabric pieces into the viable cell suspension. The same result may be obtained by filtering the suspension across the glass fabric, mounted on a suitable support.

Treatment with colloidal SiO_2-sol suspension, leading to primary entrapment of the cells in the voids of the glass fabric in accordance with step (c), is carried out with a colloidal SiO_2 suspension buffered at pH 4.0-6.5. This operation, conducted with sol suspension of aluminum hydroxide or other hydrated oxides, gives place to the same results. Different extraction rates, mentioned in step (c), are required owing to the different fiber density of the

fabric; the highest rates are used for materials with lowest fiber densities. The amount of hydokide or hydrated oxide dispersed in the sol or colloidal suspension ranges from 5 to 200 g/dm^3, and the colloidal particles may have a diameter comprised between 10 and 1000 nm.

Consolidation of the cell entrapment is performed by step (d). The choice of $Si(OR)_4$, $SiH_x(OR)_{4-x}$ and/or $SiX_x(OR)_{4-x}$, influences the adhesion of the SiO_2-like deposit, its stiffness and bulk porosity. The process is carried out in the gas phase anchoring silicon oxide species to hydroxide groups on the cell surface and the glass fabric. The solution or mixture of $Si(OR)_4$, $SiH_x(OR)_{4-x}$ and/or $SIX_x(OR)_{4-x}$ displays variable concentrations of components ranging among molar ratios $Si(OR)_4/SiH_x(OR)_{4-x}$ from 0.1/1 and 1/0.01, molar ratios $SiH_x(OR)_{4-x}/SiX_x(OR)_{4-x}$ from 0.01/2 and 1/0.1, molar ratios $Si(OR)_4/SiX_x(OR)_{4-x}$ from 1/0.01 and 0.01/1. The chemical species $SiHR(OR)_2$ where R is an alkyl or aryl, is also used in solutions or mixtures with $Si(OR)_4$ using ratios $SiHR(OR)_2/Si(OR)_4$ between 0.01/2 and 1/0.03.

The solutions or mixtures of these components are used to achieve suitable vapor pressure in the carrier gas flow. These solutions or mixtures are kept at constant temperature, variable between 20°C and 120°C, in a thermostated oil bath. The carrier gases used in this invention are air, nitrogen, argon or helium. The total flow of the gas is preferably comprised between 0.2 and 80 cm^3/minute per square centimeter of the geometrical surface of glass fabric. Treatment with vapor-phase water is carried out in a current of inert gas by bubbling the gas

into distilled water thermostated between 10 and 70°C; the total flow ranges between 0.01 and 10 cm^3/minute per cm^2 of the geometrical surface of the glass fabric.

Further characteristics and advantages of the invention will become apparent from the description of two examples, illustrated hereafter only by way of non-limitative examples with reference to the accompanying drawings:

FIGURE 1 is a photomicrograph of a glass fiber fabric, according to the invention, after coating and stabilization according to step (a);

FIGURE 2 is a photomicrograph of cells in the fabric, according to step (b);

FIGURE 3 is a drawing of the glass reactor used in step (d);

FIGURE 4 is a photomicrograph of cells immobilized in the fabric, according to step (d).

EXAMPLE 1

An ordinary fabric of glass fibers, textured by 25x25 μm meshes, was cut into disks of about 25 mm diameter. These were hydrolysed by fluxing steam for 2 hours. A 1/1 $Si(OEt)_4/CH_3SiH(OEt)_2$ ethanol solution with nominal SiO_2 concentration=100g/dm^3 was hydrolysed with stoichiometric H_2O, OR/H_2O=0.5 molar ratio, and set aside until achievement of viscosity=100 Pas.

The disks dipped into the solution were extracted at a rate of 1 mm/s. These materials, consolidated over 15 days at 40°C, show that the glass fibers are coated by a deposit of amorphous and porous SiO_2-like material still holding Si-H and $Si-CH_3$ moieties. The morphology of the coated

fabric is shown by the SEM micrograph of Fig. 1.

A cell suspension culture of Coronilla vaginalis L. (cell line 39 RAR generated from the leaf in 1991), was kept in Gamborg's basal growth B_5 medium supplemented with 3% (w/v) of sucrose, 1.3 mg/l of 2,4- dichlorophenoxyacetic acid, 0.25 mg/l of kinetin and 0.25 mg/l of naphthalenacetic acid. The pH was adjusted to 5.7 before sterilization. Cells were transferred to fresh medium at intervals of 2 weeks and maintained at 25°C on a gyratory shaker (110 rpm) in a 12-hour photoperiod. This cell suspension was used to soak the sterile disks; the operation was performed under sterile conditions, leaving the single disks in Petri dishes filled with the cell culture for 3 days on a rotary shaker at 90 rpm, at 25°C, with a 12-hour photoperiod. Cells trapped in the fabric were observed by SEM, as shown in Fig. 2.

Single disks were washed on the surface, to eliminate any non-trapped cell load. The disks were dipped into a SiO_2 sterile sol suspension. This colloidal suspension, with a particle diameter of 40 nm, was buffered at pH 5.7 with phosphatic alkaline salts and diluted with distilled water to a nominal SiO_2 concentration of 20 g/dm^3.

The disks, extracted at a rate of 1 mm s^{-1}, were mounted on a rack and introduced into the glass reactor depicted in Fig. 3. This reactor was supplied with a gas flow of air saturated by $Si(OEt)_4$ and $CH_3SiH(OEt)_2$ from a 80/20 molar ratio solution thermostated at 85°C. Total gas flow was 15 ml $minute^{-1}$ per 125 cm^2 of the geometrical surface of the disks. Treatment was continued for 3 minutes; then, using the same total gas flow, disks were

treated for 2 minutes with air saturated with steam by bubbling into water thermostated at 70°C. Cells in the glass fiber disk were observed by SEM, and appeared to be immobilized by the SiO_2-like deposit, as shown in Fig. 4. Single disks were kept in B_5 medium without hormones, at 25°C in a 12-hour photoperiod on a rotary shaker.

The retained viability of cells was determined by property of plant mitochondria to reduce tetrazolium salt (TTC), affording red formazan, easily detectable by absorption spectroscopy at 485 nm. TTC (0.5% w/v) was dissolved in sodium phosphate buffer at pH 7. The TTC solution was added to single disks and incubated without shaking for 24 hours in the dark at 23°C. The red formazan was extracted from the immobilized cells with 5 ml of 95% ethanol for 15 minutes. Cell viability was also tested by cultivating two stretched disks on solid B_5 medium supplemented with growth hormones. The induction of microcalli was used as indicator of cell culture viability.

Maintenance of immobilization was tested by controlling the occurrence of free cells in the medium of 10 disks kept at 25°C in a 12-hour photoperiod on a rotary shaker. Tests were performed every 14 days by direct microscopic observation of the solution or after 21 days' ageing of the solution supplemented with hormones.

Contamination of the solution by immobilized cells over a period of six months was checked and found to be nil.

Immobilized cells produce secondary metabolites, as coumarin compounds, since fluorescence analysis of the medium, where immobilized cells are maintained, indicates

the presence of fluorescent compounds, the concentration of which increases over four months of observation.

EXAMPLE 2

Cell suspension culture of Coronilla viminalis Salisb. (cell line 7 CFP generated from the leaf in 1991) was kept in MS medium supplemented with 3% (w/v) of sucrose, 1.3 mg/l of 2,4-dichlorophenoxyacetic acid, 0.25 mg/l of kinetin and 0.25 mg/l of naphthalenacetic acid. The pH was adjusted to 5.7 before sterilization.

Cells were transferred to fresh medium at intervals of 2 weeks and maintained at 25°C on a gyratory shaker (110 rpm) in a 12-hour photoperiod. This cell suspension was used to soak the sterile disks obtained according to the method used in example 1.

Cells were trapped and immobilized according to the method used in example 1. Single disks are maintained in MS medium without hormones, at 25°C in a 12-hour photoperiod on a rotary shaker.

Cells viability was tested by TTC reduction and by microcallus induction from stretched disks on solid MS medium supplemented with growth hormones.

No cell release from disks into the medium was observed over a period of six months.

CLAIMS

1. A process for production of secondary metabolites of viable plant cells, comprising the steps of:

(a) providing a support comprising a substantially uniform and porous matrix of inorganic material having a tensile strength of at least 500 MPa;

(b) introducing a culture of viable plant cells into the pores of said matrix;

(c) entrapping the plant cells by coating the matrix with a sol or colloidal suspension not interfering with the cell viability;

(d) immobilizing the entrapped cells within the matrix with a reactive gas including a carrier gas saturated with volatile SiO_2 or organic modified SiO_2 precursors.

2. A process as claimed in claim 1, wherein the carrier gas used in the immobilization step d) is saturated with $Si(OR)_4$, $SiH_x(OR)_{4-x}$ and/or $SiX_x(OR)_{4-x}$, wherein x=1,2, R is an alkyl or aryl, X a halide or an alkyl.

3. A process as claimed in claim 1, wherein the immobilization step d) is carried out on the surface of the entrapped plant cells in the pores of the porous support, and is followed by a treatment of the cells with vapour-phase water.

4. A process as claimed in claims 1 to 3, wherein the culture of plant cells is generated from tissues of plants maintained under chemical and physiological conditions suitable for growing biological masses.

5. A process as claimed in claim 1, wherein the introduction step (b) comprises physical methods including shaking the porous inorganic support into the cell suspension, filtering the cell suspension therethrough, migration of cells by external drivers such as magnetic or electric fields, and spontaneous introduction into the porous support by reproductive invasion.

6. A process as claimed in any preceding claims, wherein the immobilizing matrix prevents the cells from being released for a period suitable to carry out the substantially continuous production of secondary metabolites in a process plant.

7. A process as claimed in any preceding claims, wherein stimulating co-factors are added to the culture of immobilized cells to increase the production of secondary metabolites.

8. A process as claimed in any preceding claims, wherein enzymes and other chemical substances are added to the culture of immobilized cells to preserve and enhance the physiological functions of the plant cells.

9. A process as claimed in claim 1, wherein the matrix forming said support is a porous glass, ceramic, clay or similar inorganic material.

10. A process as claimed in claim 1, wherein the matrix forming said support is a fabric or an agglomerate

of inorganic fibres.

11. A process as claimed in claim 10, wherein the fabric or agglomerate of inorganic fibres is impregnated with a gelling solution of SiO_2 precursors or similar materials to increase the stiffness thereof.

12. A process as claimed in claim 11, wherein the sol or colloidal suspension is an aqueous suspension of hydroxides or hydrate oxides including SiO_2, having a pH ranging from 4.0 to 6.5 and a viscosity comprised between 0.2 and 100 Pas.

13. A process as claimed in claim 11, wherein the amount of hydokide or hydrated oxide dispersed in the sol or colloidal suspension ranges from 5 to 200 g/dm^3, and the colloidal particles have a diameter comprised between 10 and 1000 nm.

14. An article for maintaining a culture of plant cells in a stationary phase for in situ applications for the production of secondary metabolites according to any preceding claims, characterized in that it comprises a support made of a porous matrix of inorganic material having a substantially uniform distribution of pores for entrapping the cells and maintaining them in a viable condition, wherein said porous matrix has a ultimate tensile strength of at least 500 MPa.

15. Article as claimed in claim 14, wherein said

support is at least one disk-like element made of a porous matrix of glass, ceramic, fabric or glass fibres or similar inorganic materials, said at least one disk-like element being confinable within a closed reactor supplied with a flow of a reactive gas for immobilizing the viable plant cells and for production of the secondary metabolites thereof.

16. Article as claimed in claim 14, wherein the porous matrix incorporates co-factors, enzymes and/or chemical substances suitable for preserving or enhancing the physiological functions of the plant cells.

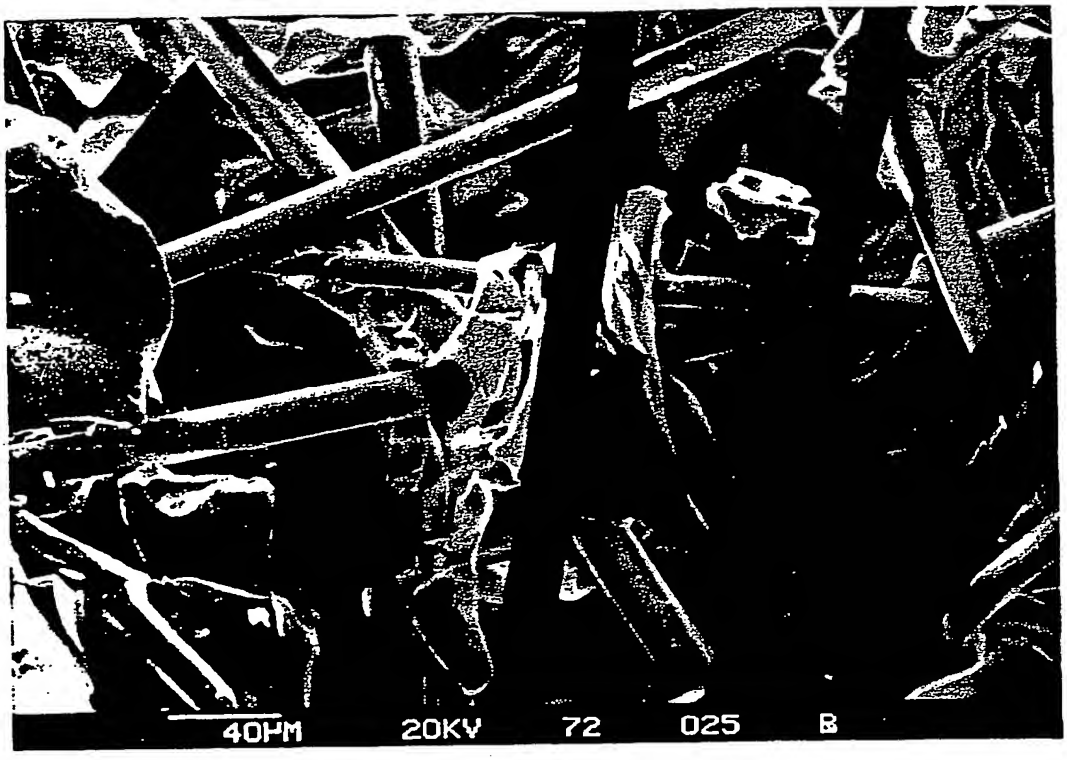

FIG. 1

FIG. 2

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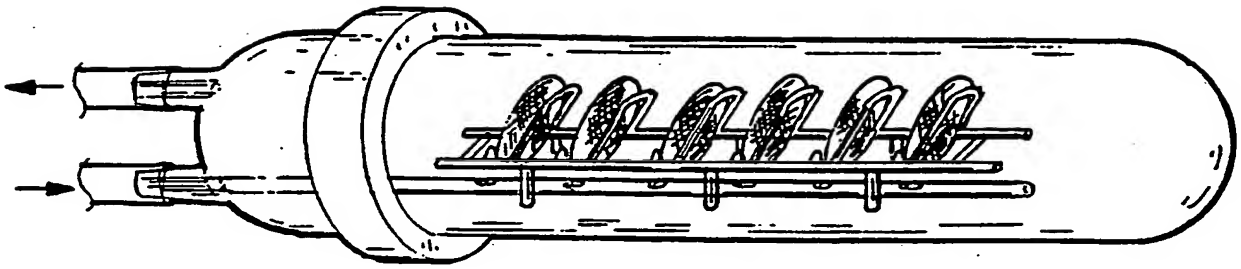

FIG. 3

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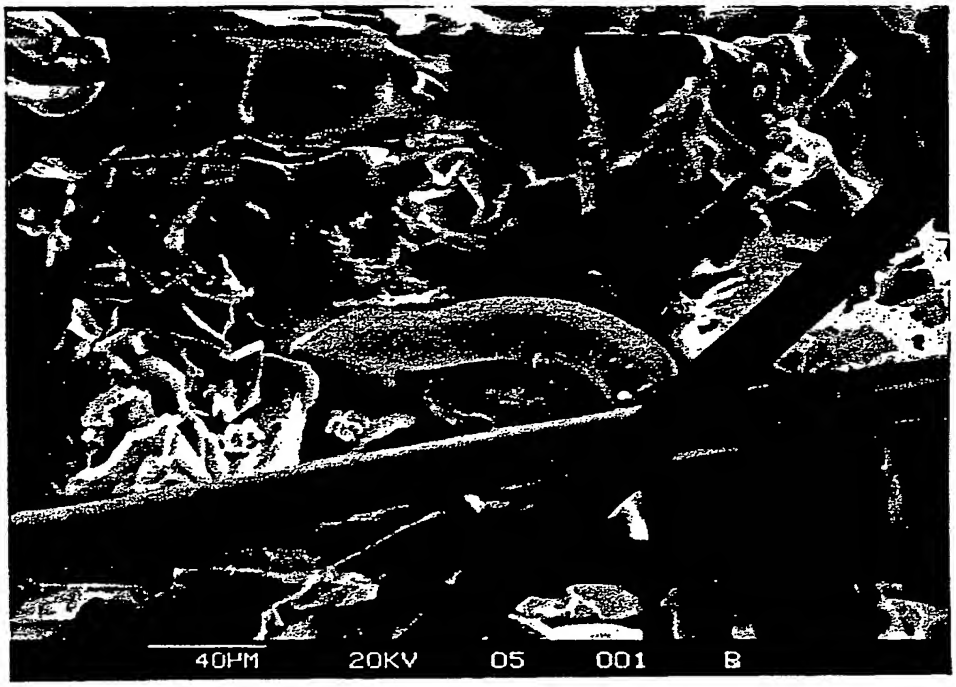

FIG. 4

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 267 470 (MANVILLE CORPORATION) 18 May 1988 see the whole document ---	14-16
A	GB,A,2 185 998 (PILKINGTON BROTHERS P.L.C.) 5 August 1987 see the whole document ---	1-16
A	JOURNAL OF BIOTECHNOLOGY, vol. 30, 1993 AMSTERDAM, pages 197-210, L. INAMA ET AL 'Entrapment of viable microorganisms by SiO2 sol-gel layers on glass surfaces: Trapping, catalytic performance and immobilization durability of Saccharomices cerevisiae' see the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAN HLAVAC 'The technology of glass and ceramics' 1983 , ELSEVIER SCIENTIFIC PUBLISHING COMPANY , AMSTERDAM see page 173 - page 177	14-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Appl on No
PCT/IT 95/00083

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GB-A-2185998	05-08-87	NONE		